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(54) Title: GROUP A STREPTOCOCCAL VACCINES

SphI	BamHII	Sall	Ncol	PstI	KpnI	ClaI	HindIII
5' M24	M5	M6	M19	M1.0	M3	M24	3'

60 Codons **58 Codons** **35 Codons ex.** **30 Codons** **50 Codons** **80 Codons**

(57) Abstract

The present invention provides immunogenic synthetic fusion polypeptide which stimulates an immune response against a selected pathogen, comprising at least two immunogenic polypeptides from a Group A streptococci of at least 10 amino acids in length which are capable of stimulating an immune response against Group A streptococci, and a peptide C terminal to the immunogenic polypeptide which protects the immunogenicity of the immunogenic portion, wherein the C-terminal peptide is not required to stimulate an immune response against Group A streptococci.

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GROUP A STREPTOCOCCAL VACCINES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of United States Provisional Application No. 60/058,635, filed September 12, 1997, which application is incorporated by reference in its entirety.

TECHNICAL FIELD

The present invention provides pharmaceutical compositions and methods, and in particular, vaccines for use in preventing Group A streptococcal infections.

BACKGROUND OF THE INVENTION

Streptococci are a group of bacteria with the capacity to grow in chains. Many varieties are part of the normal bacterial flora in humans and are not especially harmful. However, a particular group of streptococcal bacteria, called group A and represented by *Streptococcus pyogenes*, is a human pathogen. Briefly, group A streptococci cause a variety of human illnesses, ranging from uncomplicated pharyngitis and pyoderma to life-threatening infections associated with toxic shock syndrome, deep tissue invasion and sepsis. In some individuals, untreated streptococcal pharyngitis may be followed by acute rheumatic fever. In recent years there has been a dramatic increase in the incidence of severe streptococcal infections (Davies et al., "Invasive group A streptococcal infections in Ontario, Canada. Ontario group A Streptococcal study group," *N. Engl. J. Med.* 335:547-554, 1996) and in the incidence of rheumatic fever (Veasey et al., "Resurgence of acute rheumatic fever in the intermountain region of the United States," *N. Eng. J. Med.* 316:421-427, 1987).

Although streptococcal infections can be generally treated with antibiotics, in at least 4% of cases the infection leads to acute rheumatic fever. This disease is particularly prevalent in developing countries such as India, where millions of school-age children are affected.

The present invention provides new Group A streptococcal vaccines with enhanced immunogenicity, and further, provides other related advantages.

SUMMARY OF THE INVENTION

Briefly stated, the present invention provides immunogenic synthetic fusion polypeptides which stimulate an immune response against Group A streptococci. Within one aspect such polypeptides comprise (a) at least two immunogenic polypeptides from a Group A streptococci of at least 10 amino acids in length which are capable of stimulating an immune response against Group A streptococci, and a peptide C terminal to the immunogenic polypeptide which protects the immunogenicity of the immunogenic portion. Within preferred embodiments, the C-terminal peptide is not required to stimulate an immune response against Group A streptococci and hence, may be an inconsequential non-immunogenic peptide, or a reiterated immunogenic polypeptide. Within certain embodiments, the immunogenic polypeptide can be obtained from a wide variety of Group A streptococci (ranging from "1" to greater than "90"), including for example, Types 1, 1.1, 2, 3, 4, 5, 6, 11, 12, 13, 14, 18, 19, 22, 24, 28, 30, 48, 49, 52, 55 and 56.

Within other aspects of the present invention, vaccinating agents are provided for promoting an immune response against Group A streptococci, comprising (a) at least two immunogenic polypeptides from a Group A streptococci of at least 10 amino acids in length which are capable of stimulating a protective immune response against Group A streptococci, and (b) a peptide C terminal to the immunogenic polypeptide which protects the immunogenicity of the immunogenic portion, wherein the C-terminal peptide is not required to stimulate an immune response against Group A streptococci. As above, the polypeptide may be selected from a wide variety of Group A streptococci (ranging from "1" to greater than "90"), including for example, types 1.1, 2, 3, 4, 5, 6, 11, 12, 13, 14, 18, 19, 22, 24, 28, 30, 48, 49, 52, 55 and 56. Within certain further embodiments, the vaccinating agent may further comprise an adjuvant, such as, for example, alum, Freund's adjuvant, and/or an immunomodulatory cofactor (e.g., IL-4, IL-10, γ -IFN, or IL-2, IL-12 or IL-15).

Also provided are methods for vaccinating a host against Group A streptococci infections, comprising administering a vaccinating agent as described above.

These and other aspects of the present invention will become evident 5 upon reference to the following detailed description and attached drawings. In addition, various references are set forth herein which describe in more detail certain procedures or compositions (e.g., plasmids, etc.), and are therefore incorporated by reference in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS

10 Figure 1 is a schematic of the hexavalent vaccine indicating the length of each emm gene fragment and the restriction sites that were synthesized into the original PCR primers. Each of the emm gene fragments starts at the first codon that encodes the mature native protein except the emm3 fragment, which represents codons 21-70.

15 Figure 2 is a SDS-polyacrylamide gel electrophoresis of the purified hexavalent protein stained with coomassie blue. Computer-assisted image analysis of the stained protein bands indicated that the hexavalent protein (M.W. 45 kDa) accounted for 90.3% of the total protein in the sample.

20 Figures 3A and 3B are ELISA's of antisera from three rabbits immunized with the hexavalent vaccine in either alum or CFA. Titers are expressed as the inverse of the last dilution of serum that resulted in an O.D. of >0.1. The ELISA antigen was the purified hexavalent protein. Each symbol represents serum from one rabbit.

25 Figures 4A-4F are ELISA's of antisera from rabbits immunized with the hexavalent protein in alum. Titers were determined using the purified pepsin-extracted M proteins (pep M) from the respective serotypes of group A streptococci. Each symbol represents one of three immunized rabbits.

30 Figure 5 depicts *in vitro* opsonization assays of antisera from rabbits immunized with the hexavalent protein in alum. Rotation mixtures consisted of the test organism, 0.1 ml of immune serum, and 0.4 ml of nonimmune human blood. The mixture was rotated for 45 minutes and the percentage of PMNs that had

ingested or were associated with streptococci was estimated by microscopic counts of stained smears. In each assay, the preimmune serum resulted in <10% percent opsonization. Each different bar represents serum from one of the three immunized rabbits.

5 Figure 6 is a graph which depicts opsonization of different strains within the same serotype of group A streptococci promoted by hexavalent rabbit antisera. Each symbol represents a strain of group A streptococci of the serotype indicated on the horizontal axis. Opsonization assays were performed as described below in the Examples.

10 Figure 7 is a sequence of the hexavalent M protein vaccine (SEQ ID NO:15 and SEQ ID NO:16).

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

Prior to setting forth the invention, it may be helpful to an
15 understanding thereof to first set forth definitions of certain terms that will be used hereinafter.

"Vaccinating Agent" refers to a composition which is capable of stimulating a protective immune response within the host which receives the vaccinating agent. The vaccinating agent may be either protein, or, DNA-based (*e.g.*,
20 a gene delivery vehicle). Within further aspects, a prokaryotic host may be generated to be a vaccinating agent, and designed to express an immunogenic polypeptide or multivalent construct of the present invention (*see, e.g.*, U.S. Application No. 07/540,586).

"Gene delivery vehicle" refers to a recombinant vehicle, such as a
25 recombinant viral vector, a nucleic acid vector (such as plasmid), a naked nucleic acid molecule such as genes, a nucleic acid molecule complexed to a polycationic molecule capable of neutralizing the negative charge on the nucleic acid molecule and condensing the nucleic acid molecule into a compact molecule, a nucleic acid associated with a liposome (Wang et al., *PNAS* 84:7851, 1987), a bacterium, and
30 certain eukaryotic cells such as a producer cell, that are capable of delivering a

nucleic acid molecule having one or more desirable properties to host cells in an organism.

As noted above, the present invention provides vaccinating agents suitable for preventing Group A streptococcal infections. Briefly, as described in 5 more detail below it has been discovered that, in order to optimize the immunogenicity of all aspects of a multivalent vaccine. Within one aspect of the invention, immunogenic synthetic fusion polypeptides which stimulate an immune response against Group A streptococci are provided. Such polypeptides generally comprise (a) at least two immunogenic polypeptides from a Group A streptococci of at 10 least 10 amino acids in length which are capable of stimulating an immune response against Group A streptococci, and (b) a peptide C terminal to the immunogenic polypeptide which protects the immunogenicity of the immunogenic portion, wherein the C-terminal peptide is not required to stimulate an immune response against Group A streptococci. Particularly preferred protective peptides are generally at least ten 15 amino acids in length, and may be 30 amino acids or longer.

Identification of Immunogenic Polypeptides for Use in Vaccinating Agents

Immunogenic polypeptides suitable for use within the present invention may be readily identified and generated given the disclosure of the subject application (*see also* Dale and Beachey, *J. Exp. Med.* 163:1191-1202; 1986; Beachey 20 et al., *Nature* 292:457-459, 1981; Dale et al., *J. Immunol.* 151:2188-2194; 1993; and U.S. Patent Nos. 4,454,121; 4,521,334; 4,597,967; 4,705,684; 4,919,930; and 5,124,153). Particularly preferred polypeptides can be obtained within the 50 amino acid residues of the N-terminus of an M protein.

Serotypes of Group A streptococci can be readily obtained from 25 clinical isolates, from university collections (e.g., Rockefeller University Collection, 1230 York Avenue, New York, NY) or from depositories such as the American Type Culture Collection (10801 University Boulevard, Manassas, Virginia). Furthermore, sequences for Group A streptococci serotypes are available from the Centers for Disease Control, Atlanta, Georgia.

A. Identification of Opsonic Epitopes of M Proteins

To demonstrate directly that opsonic M protein epitopes could be separated from autoimmune epitopes, peptides are copied from various serotypes (e.g., the amino-terminal 20-50 amino acids of M5 (Beachey et al., "Purification and properties of M protein extracted from group A streptococci with pepsin: Covalent structure of the amino terminal region of the type 24 M antigen," *J. Exp. Med.* 145:1469-1483, 1977). SM5(1-20) failed to react with affinity purified pep M5 heart-reactive antibodies (Beachey et al., "Purification and properties of M protein extracted from group A streptococci with pepsin: Covalent structure of the amino 5 terminal region of the type 24 M antigen," *J. Exp. Med.* 145:1469-1483, 1977). Rabbits immunized with SM5(1-20) coupled to tetanus toxoid developed high titers of antibodies against pep M5 that opsonized type 5 streptococci (Beachey et al., "Purification and properties of M protein extracted from group A streptococci with pepsin: Covalent structure of the amino terminal region of the type 24 M antigen," *J. 10 Exp. Med.* 145:1469-1483, 1977). Most importantly, none of the immune sera 15 crossreacted with human myocardium.

B. Tissue-Crossreactive Epitopes of M Proteins

M proteins evoke antibodies that crossreact with a variety of human 20 tissues and antigens within those tissues (Baird et al., "Epitopes of group A streptococcal M protein shared with antigens of articular cartilage and synovium," *J. Immunol.* 146:3132-3137, 1991; Bronze, M.S. and Dale, J.B., "Epitopes of streptococcal M proteins that evoke antibodies that cross-react with human brain," *J. 25 Immunol.* 151:2820-2828., 1993; Dale, J.B. and Beachey E.H., "Protective antigenic determinant of streptococcal M protein shared with sarcolemmal membrane protein of human heart," *J. Exp. Med.* 156:1165-1176, 1982). In order to determine cross-reactivity, a series of overlapping peptides is synthesized that copies a selected fragment (e.g., M5), and used to either inhibit or evoke tissue-crossreactive antibodies. For example, the myosin-crossreactive antibodies evoked by pep M5 in 30 rabbits were almost totally inhibited by peptide 84-116 of pep M5. This peptide spans the region between the A and B repeats of M5 and includes the degenerate A6

repeat. Murine and human myosin-crossreactive antibodies reacted with an epitope in peptide 183-189, which is located in the region between the B and C repeats of the intact M5 molecule.

Additional sarcolemmal membrane crossreactive epitopes are 5 localized to peptide 164-197. Several epitopes of M5 that evoked antibodies that crossreacted with articular cartilage and synovium can also be found within the B repeats and the region spanning the A and B repeats of M5. The brain-crossreactive epitopes of M6 that were shared with other M proteins are localized to the B repeat region of the molecule.

10 Many of the tissue-crossreactive epitopes are shared among types 5, 6, 18 and 19 M proteins (Bronze, M.S. and Dale, J.B., "Epitopes of streptococcal M proteins that evoke antibodies that cross-react with human brain," *J. Immunol.* 151:2820-2828., 1993). Primary structural data reveals that all of these M proteins contain similar sequences within their B repeats (Dale et al., "Recombinant 15 tetravalent group A streptococcal M protein vaccine," *J. Immunol.* 151:2188-2194, 1993; Dale et al., "Recombinant, octavalent group A streptococcal M protein vaccine," *Vaccine* 14:944-948, 1996; Dale et al., "Type-specific immunogenicity of a chemically synthesized peptide fragment of type 5 streptococcal M protein," *J. Exp. Med.* 158:1727-1732, 1983), which is most likely the location of the shared heart-, 20 brain-, and joint-crossreactive epitopes.

It should be emphasized that it is not necessary to localize the tissue-specific epitope, but rather, to first localize protective epitopes and ensure that they are not tissue-reactive.

Once a suitable immunogenic polypeptide for a selected serotype has 25 been identified, it may be, optionally, combined with immunogenic polypeptides from other serotypes, in order to construct a multivalent vaccine. In this regard, preferred vaccines include vaccines developed from a combination of serotypes such as 1, 1.1, 2, 3, 4, 5, 6, 11, 12, 13, 14, 18, 19, 22, 24, 28, 30, 48, 49, 52 and 56 (for serotype 30 see Nakashima et al., *Clinic Infect. Dis.* 25:260, 1997). Representative 30 examples include vaccine such as 24, 5, 6, 19, 1, 3, X; and 1, 3, 5, 6, 18, 19, 22, 24, 28, 30, and X, wherein X is the C-terminal protective polypeptide.

PREPARATION OF VACCINATING AGENTS

Vaccinating agents of the present invention can be synthesized chemically (see, e.g., Beachey et al., *Nature* 292:457-459, 1981), or generated recombinantly. For recombinant production, PCR primers can be synthesized to 5 amplify desired 5' sequences of each emm gene, and each primer is extended to contain a unique restriction enzyme site used to ligate the individual PCR products in tandem.

As noted above, the C-terminal portion of the vaccinating agent is constructed so as to contain a selective portion that can be lost or cleaved *in vivo* 10 without affecting the efficacy of the vaccine. This may be accomplished by, for example, including an inconsequential non-immunogenic polypeptide at the end, or, including an immunogenic polypeptide that does not adversely impact the efficiency of the vaccine (e.g., a reiterated immunogenic polypeptide may be included at the end of the vaccine). Furthermore, protective antigens from unrelated pathogens can also 15 be combined into a single polypeptide, which may circumvent the need for carriers. Vaccines against some pathogens might include T and B cell epitopes originally derived from different proteins on the same hybrid construct. Additionally, multivalent hybrid proteins may be sufficient conjugates in carbohydrate vaccines, such as those for *S. pneumoniae*, *H. influenza* B or group B streptococci.

For protein expression, the multivalent genes are ligated into any 20 suitable replicating plasmid which is used to transform an appropriate prokaryote host strain. Prokaryotes include gram negative or gram positive organisms, for example *E. coli* or bacilli. Suitable prokaryotic hosts cells for transformation include, for example, *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium*, and various other 25 species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*.

Expression vectors transfected into prokaryotic host cells generally comprise one or more phenotypic selectable markers such as, for example, a gene 30 encoding proteins that confer antibiotic resistance or that supplies an autotrophic requirement, and an origin of replication recognized by the host to ensure amplification within the host. Other useful expression vectors for prokaryotic host cells include a selectable marker of bacterial origin derived from commercially available plasmids. This selectable marker can comprise genetic elements of the

cloning vector pBR322 (ATCC 37017). Briefly, pBR322 contains genes for ampicillin and tetracycline resistance and thus provides simple means for identifying transformed cells. The pBR322 "backbone" sections are combined with an appropriate promoter and a mammalian ETF structural gene sequence. Other 5 commercially available vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden), pQE30 and pGEM1 (Promega Biotec, Madison, WI, USA).

Common promoter sequences for use within prokaryotic expression vectors include β -lactamase (penicillinase), lactose promoter system (Chang et al., 10 *Nature* 275:615, 1978; and Goeddel et al., *Nature* 281:544, 1979), tryptophan (trp) promoter system (Goeddel et al., *Nucl. Acids Res.* 8:4057, 1980; and EPA 36,776) and tac promoter (Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, (1989)). A particularly useful prokaryotic host cell expression system employs a phage λ P_L promoter and a *cI857ts* thermolabile 15 repressor sequence. Plasmid vectors available from the American Type Culture Collection that incorporate derivatives of the λ P_L promoter include plasmid pHUB2 (resident in *E. coli* strain JMB9 (ATCC 37092)) and pPLc28 (resident in *E. coli* RR1 (ATCC 53082)).

Transformation of the host strains of *E. coli* is accomplished by 20 electroporation using standard methods (Dale et al., "Recombinant tetravalent group A streptococcal M protein vaccine," *J. Immunol.* 151:2188-2194, 1993; Dale et al., "Recombinant, octavalent group A streptococcal M protein vaccine," *Vaccine* 14:944-948, 1996). Successful transformants are identified by colony blots using rabbit antisera raised against one of the native M proteins or a synthetic peptide copy 25 of the amino-terminus of one of the M proteins included in the multivalent protein.

The molecular size and antigenicity of the recombinant protein expressed by selected clones are determined by performing Western blots of extracts of *E. coli* (Dale et al., "Recombinant tetravalent group A streptococcal M protein vaccine," *J. Immunol.* 151:2188-2194, 1993) using rabbit antisera raised against each 30 native M protein purified from pepsin extracts of live streptococci (Beachey et al., "Purification and properties of M protein extracted from group A streptococci with pepsin: Covalent structure of the amino terminal region of the type 24 M antigen," *J.*

Exp. Med. 145:1469-1483, 1977). The multivalent gene is sequenced by the dideoxy-nucleotide chain termination method to confirm that each gene fragment is an exact copy of the native emm sequence.

5

GENE-DELIVERY VEHICLE-BASED VACCINES

Injection of mammals with gene delivery vehicles (e.g., naked DNA) encoding antigens of various pathogens has been shown to result in protective immune responses (Ulmer et al., *Science* 259:1745-9, 1993; Bourne et al., *J Infect. Dis.* 173:800-7, 1996; Hoffman et al., *Vaccine* 12:1529-33, 1994). Since the original 10 description of *in vivo* expression of foreign proteins from naked DNA injected into muscle tissue (Wolff et al., *Science* 247:1465-8, 1990), there have been several advances in the design and delivery of DNA for purposes of vaccination.

The M protein vaccines described above are ideally suited for delivery via naked DNA because protective immunity is ultimately determined by antibodies. 15 For example, within one embodiment the multivalent genes are ligated into plasmids that are specifically engineered for mammalian cell expression (see, e.g., Hartikka et al., *Hum Gene Ther* 7:1205-17, 1996, which contains the promoter/enhancer element from cytomegalovirus early gene, the signal peptide from human tissue plasminogen activator and a terminator element from the bovine growth hormone gene). The M 20 protein hybrid genes can be cloned into the plasmid which is used to transfect human cell lines to assure recombinant protein expression. The plasmid is propagated in *E. coli* and purified in quantities sufficient for immunization studies by cesium chloride gradient centrifugation. Mice are immunized with 50 ug of plasmid in 50 ul saline given intramuscularly into the rectus femoris. Booster injections of the same dose 25 are given at three and six weeks after the initial injection.

A wide variety of other gene delivery vehicles can likewise be utilized within the context of the present invention, including for example, viruses, retrotransposons and cosmids. Representative examples include adenoviral vectors (e.g., WO 94/26914, WO 93/9191; Yei et al., *Gene Therapy* 1:192-200, 1994; Kolls 30 et al., *PNAS* 91(1):215-219, 1994; Kass-Eisler et al., *PNAS* 90(24):11498-502, 1993; Guzman et al., *Circulation* 88(6):2838-48, 1993; Guzman et al., *Cir. Res.* 73(6):1202-1207, 1993; Zabner et al., *Cell* 75(2):207-216, 1993; Li et al., *Hum Gene*

Ther. 4(4):403-409, 1993; Caillaud et al., *Eur. J. Neurosci.* 5(10):1287-1291, 1993), adeno-associated type 1 ("AAV-1") or adeno-associated type 2 ("AAV-2") vectors (see WO 95/13365; Flotte et al., *PNAS* 90(22):10613-10617, 1993), hepatitis delta vectors, live, attenuated delta viruses and herpes viral vectors (e.g., U.S. Patent No. 5,288,641), as well as vectors which are disclosed within U.S. Patent No. 5,166,320. Other representative vectors include retroviral vectors (e.g., EP 0 415 731; WO 90/07936; WO 91/02805; WO 94/03622; WO 93/25698; WO 93/25234; U.S. Patent No. 5,219,740; WO 93/11230; WO 93/10218). Methods of using such vectors in gene therapy are well known in the art, see, for example, Larrick, J.W. and Burck, K.L., *Gene Therapy: Application of Molecular Biology*, Elsevier Science Publishing Co., Inc., New York, New York, 1991; and Kreigler, M., *Gene Transfer and Expression: A Laboratory Manual*, W.H. Freeman and Company, New York, 1990.

Gene-delivery vehicles may be introduced into a host cell utilizing a vehicle, or by various physical methods. Representative examples of such methods include transformation using calcium phosphate precipitation (Dubensky et al., *PNAS* 81:7529-7533, 1984), direct microinjection of such nucleic acid molecules into intact target cells (Acsadi et al., *Nature* 352:815-818, 1991), and electroporation whereby cells suspended in a conducting solution are subjected to an intense electric field in order to transiently polarize the membrane, allowing entry of the nucleic acid molecules. Other procedures include the use of nucleic acid molecules linked to an inactive adenovirus (Cotton et al., *PNAS* 89:6094, 1990), lipofection (Felgner et al., *Proc. Natl. Acad. Sci. USA* 84:7413-7417, 1989), microprojectile bombardment (Williams et al., *PNAS* 88:2726-2730, 1991), polycation compounds such as polylysine, receptor specific ligands, liposomes entrapping the nucleic acid molecules, spheroplast fusion whereby *E. coli* containing the nucleic acid molecules are stripped of their outer cell walls and fused to animal cells using polyethylene glycol, viral transduction, (Cline et al., *Pharmac. Ther.* 29:69, 1985; and Friedmann et al., *Science* 244:1275, 1989), and DNA ligand (Wu et al., *J. of Biol. Chem.* 264:16985-16987, 1989), as well as psoralen inactivated viruses such as Sendai or Adenovirus.

Serum from mice immunized with gene delivery vehicles containing multivalent M protein genes are assayed for total antibody titer by ELISA using

native M proteins as the antigen. Serum opsonic antibodies are assayed as described above. Protective efficacy of DNA M protein vaccines is determined by direct mouse protection tests using the serotypes of group A streptococci represented in the vaccine.

5

FORMULATION AND ADMINISTRATION

For therapeutic use, vaccinating agents can be administered to a patient by a variety of routes, including for example, by intramuscular, subcutaneous, and mucosal routes. The vaccinating agent may be administered as a single dosage, 10 or in multiple units over an extended period of time. Within preferred embodiments, the vaccinating agent is administered to a human at a concentration of 50-300 ug per single site intramuscular injection. Several injections can be given (e.g., three or four) at least one month apart in order to further increase vaccine efficacy.

Typically, the vaccinating agent will be administered in the form of a 15 pharmaceutical composition comprising purified polypeptide in conjunction with physiologically acceptable carriers, excipients or diluents. Such carriers will be nontoxic to patients at the dosages and concentrations employed. Ordinarily, the preparation of such compositions entails combining the vaccinating agent with buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose, 20 sucrose or dextrans, chelating agents such as EDTA, glutathione and other stabilizers and excipients. Neutral buffered saline or saline mixed with conspecific serum albumin are exemplary appropriate diluents.

Within preferred embodiments of the invention, the vaccinating agent 25 is combined with an adjuvant, such as, for example, Freund's adjuvant, alum and the like.

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLES

EXAMPLE 1

CONSTRUCTION AND EXPRESSION OF A HEXAVALENT FUSION GENE

5

A hexavalent emm gene was constructed using PCR to amplify specific 5' regions of the six different emm genes (24, 5, 6, 19, 1 and 3) essentially as described previously (Dale et al., "Recombinant tetravalent group A streptococcal M protein vaccine," *J. Immunol.* 151:2188-2194, 1993; Dale et al., "Recombinant, 10 octavalent group A streptococcal M protein vaccine," *Vaccine* 14:944-948, 1996).

Briefly, the multivalent genes are constructed using PCR and primers that specify specific 5' emm gene fragments. The gene fragments may range in size from 30 bp to 300 bp. Chromosomal DNA from each serotype of group A streptococcus is used as the template for the PCR reactions. For the hexavalent emm 15 gene described in the example, the PCR primers are as follows:

M24-1 TS SphI
5' GGG GGG GCA TCG GTC GCG ACT AGG TCT CAG ACA GAT 3'
(SEQ ID NO:1)

M24-1 BS BamH1
5' GGG GGG GGA TCC ACG TAG TTT CTC TTT AGC 3'
(SEQ ID NO:2)

M5 TS BamH1
5' GGG GGG GGA TCC GCC GTG ACT AGG GGT ACA 3'
(SEQ ID NO:3)

M5 BS SalI
5' GGG GGG GTC GAC CTC AGT TTT TAA CCC TTC 3'
(SEQ ID NO:4)

M6 TS SalI
5' GGG GGG GTC GAC AGA GTG TTT CCT AGG GGG 3'
(SEQ ID NO:5)

M6 BS NcoI
5' GGG GGG CCA TGG TAA CTT GTC ATT ATT AGC 3'
(SEQ ID NO:6)

40

M19 TS NcoI
5' GGG GGG CCA TGG AGA GTG CGT TAT ACT AGG 3'
(SEQ ID NO:7)

5 M19 BS PstI
5' GGG GGG CTG CAG AGA TAA CTT CTC ATT CTG 3'
(SEQ ID NO:8)

10 M1 TS PstI
5' GGG GGG CTG CAG AAC GGT GAT GGT AAT CCT 3'
(SEQ ID NO:9)

15 M1 BS KpnI
5' GGG GGG GGT ACC AGC TCT CTT AAA ATC TCT 3'
(SEQ ID NO:10)

20 M3 TS KpnI
5' GGG GGG GGT ACC TTG TTA GAT CAG GTT ACA 3'
(SEQ ID NO:11)

M3 BS Clal
5' GGG GGG ATC GAT ATT TAA CTC TTG TAA CAG 3'
(SEQ ID NO:12)

25 M24-2 TS Clal
5' GGG GGG ATC GAT GTC GCG ACT AGG TCT CAG 3'
(SEQ ID NO:13)

30 M24-2 BS HindIII
5' GGG GGG AAG CTT TTA CTT ACG TGC CTC TAA TTC 3'
(SEQ ID NO:14)

PCR is performed on the chromosomal template as previously described (Dale et al., "Recombinant tetravalent group A streptococcal M protein vaccine," *J. Immunol.* 151:2188-2194, 1993). To assure ligation of the fragments in the correct orientation and reading frame, each PCR product is purified, ligated, and then subjected to PCR again using the forward primer from the 5' fragment and the reverse primer from the 3' fragment. For example, to construct a hexavalent emm gene containing DNA sequences from types 24, 5, 6, 19, 1, and 3 M proteins, the 35 M24 and M5 gene fragments are amplified by PCR using the primers described above. The PCR products are purified from agarose gels, cut with the appropriate restriction enzyme, and ligated together (Dale et al., "Recombinant tetravalent group 40 A streptococcal M protein vaccine," *J. Immunol.* 151:2188-2194, 1993; Dale et al.,

"Recombinant, octavalent group A streptococcal M protein vaccine," *Vaccine* 14:944-948, 1996). The ligation mixture is then amplified by PCR using the forward M24 primer and the reverse M5 primer. The resulting product of the appropriate size is then purified and ligated to the M6 and M19 gene fragment that was similarly constructed. After the final ligation reaction, the entire gene is amplified again by PCR, cut with the appropriate restriction enzymes and ligated into a suitable expression vector. For the addition of the reiterated M24 gene fragment in the 3' location, the plasmid was purified from the host *E. coli* and a new PCR product from emm 24 was force cloned into the 3' PstI restriction site.

10 The hexavalent gene was sequenced by the dideoxy-nucleotide chain termination method to confirm that each gene fragment was an exact copy of the respective native emm sequence.

EXAMPLE 2

15

PURIFICATION OF A HEXAVALENT VACCINE

A. Purification

Transformed *E. coli* were grown in a shaking incubator to log phase in 11 of LB containing 100 ug/ml ampicillin and 25 ug/ml kanamycin. IPTG (2mM) was added for the final four hours of growth. The cell pellet was suspended in 30 ml PBS and lysed in a French pressure cell at 1000 psi. The hexavalent protein was purified from the supernatant using Ni-NTA resin according to the protocol provided by the manufacturer (Qiagen). The elution buffer containing the protein was concentrated from 15 ml to 5 ml in a spin filter (Ultrafree-15, Millipore). Final purification was accomplished by gel filtration over Superdex 75 (prep grade, 20 Pharmacia Biotech). The active fraction was identified by Western blots (Dale, J.B. and Beachey, E.H., "Multiple heart-cross-reactive epitopes of streptococcal M proteins," *J. Exp. Med.* 161:113-122, 1985) using rabbit antiserum against pep M24 (Beachey et al., "Purification and properties of M protein extracted from group A streptococci with pepsin: Covalent structure of the amino terminal region of the type 25 24 M antigen," *J. Exp. Med.* 145:1469-1483, 1977). Total protein concentration was determined by standard methods and the sample was diluted in PBS to contain 200

ug/ml of hexavalent protein. Purity of the samples was determined by gel scanning (Photoshop digital image and Collage image analysis).

B. Analysis of the hexavalent vaccine

The structure of the hybrid emm gene was confirmed by double-stranded sequencing methods after ligation into pQE30. The sequence of each subunit was identical to the respective native emm gene (Figure 1). The fragments were joined only by the two amino acids specified by each unique restriction site used to facilitate their ligation (Figure 1).

The purified hexavalent protein migrated on SDS-polyacrylamide gels with an apparent M.W. of 45 kDa. (Figure 2). Gel scan analysis revealed that the intact hexavalent protein accounted for approximately 90% of the total stainable protein in the gel. Western blots using antisera against pep M24 showed that the majority of the remaining protein bands were immunoreactive and most likely were fragments of the hexavalent protein (data not shown).

15

EXAMPLE 3

IMMUNIZATION OF RABBITS, AND TESTING OF ANTISERA

A. Immunization

20 Two groups of three rabbits each were immunized with 100 ug of hexavalent vaccine either precipitated with alum or emulsified in complete Freund's adjuvant. For precipitation in alum, the hexavalent protein (200 ug/ml) was added to an equal volume of aluminum hydroxide (2mg/ml) (Rehydragel HPA, Reheis, Inc., Berkeley Heights, NJ) and mixed gently at 40C overnight. The hexavalent protein
25 was also emulsified in CFA at a final concentration of 100 ug/ml. Rabbits that received the hexavalent vaccine in alum were given 100 ug I.M. as an initial injection and the same dose was repeated at 4, and 8 weeks. The second set of rabbits received 100 ug of hexavalent vaccine in CFA subcutaneously as an initial injection and then booster injections of the same dose in saline were given at 4 and 8 weeks. Blood was
30 obtained prior to the first injection and at 2-week intervals thereafter.

Antibody assays. ELISAs were performed using purified native pepsin-extracted M proteins (Beachey et al., "Purification and properties of M protein extracted from group A streptococci with pepsin: Covalent structure of the amino terminal region of the type 24 M antigen," *J. Exp. Med.* 145:1469-1483, 1977) or the 5 purified hexavalent protein, as previously described (Dale et al., "Heterogeneity of type-specific and cross-reactive antigenic determinants within a single M protein of group A streptococci," *J. Exp. Med.* 151:1026-1038, 1980). Opsonic antibodies were detected by *in vitro* opsonization assays and indirect bactericidal assays (Beachey et al., "Human immune response to immunization with a structurally defined 10 polypeptide fragment of streptococcal M protein," *J. Exp. Med.* 150:862-877, 1979).

B. Detection of M protein antibodies.

The preimmune and immune animal sera are assayed by ELISA using the vaccine protein and the native pepsin-extracted M proteins as solid-phase antigens (Dale et al., "Heterogeneity of type-specific and cross-reactive antigenic determinants within a single M protein of group A streptococci," *J. Exp. Med.* 151:1026-1038, 1980). ELISA titers are defined as the inverse of the last dilution of antisera resulting in an OD of >0.1 at 450nm. The titers of immune sera against the native M antigen are most likely to predict the levels of antibodies that are evoked by the recombinant protein that will react with the M protein on the surface of the 20 respective serotype of streptococcus (*i.e.* promote opsonization).

C. Detection of opsonic antibodies.

Opsonic M protein antibodies correlate with protection against infection with the same serotype of group A streptococci (Lancefield, R.C., "Current knowledge of the type specific M antigens of group A streptococci," *J. Immunol.* 89:307-313, 1962; Lancefield, R.C., "Persistence of type-specific antibodies in man 25 following infection with group A streptococci," *J. Exp. Med.* 110:271-282, 1959). Two related *in vitro* assays are used to detect opsonic antibodies in immune sera. The first is a screening assay that measures opsonization in mixtures of immune serum, whole, nonimmune human blood and the test organism (Beachey et al., 30 "Purification and properties of M protein extracted from group A streptococci with

pepsin: Covalent structure of the amino terminal region of the type 24 M antigen," *J. Exp. Med.* 145:1469-1483, 1977). 0.1 ml of test serum is added to a standard number of bacteria and incubated for 15 minutes at room temperature. 0.4 ml of lightly heparinized human blood is added and the entire mixture is rotated end-over-end at 5 370 for 45 minutes. At the end of the rotation, smears are prepared on microscope slides that are air-dried and stained with Wright's stain. "Percent opsonization" is quantitated by counting the percentage of polymorphonuclear leukocytes that have ingested or are associated with bacteria. An interpretable assay must have a preimmune control value that is 10% opsonization or less.

10 Confirmation of the presence of opsonic antibodies is obtained by indirect bactericidal antibody assays according to the original description by Lancefield (Lancefield, R.C., "Current knowledge of the type specific M antigens of group A streptococci," *J. Immunol.* 89:307-313, 1962). This assay is performed using test mixtures as described above except that fewer bacteria are added and the 15 rotation is allowed to proceed for 3 hours. At the end of the rotation, pour plates are made in sheep blood agar and bacteria surviving are quantitated after overnight growth at 370. Percent killing in the presence of immune serum is calculated by comparing to the growth in nonimmune serum.

20

EXAMPLE 4
MOUSE PROTECTION ASSAYS

A. General Protocol

Protective efficacy of M protein vaccines is determined by either 25 indirect or direct (passive or active immunization) mouse protection tests. Indirect tests are performed by giving mice 1 ml of immune or preimmune serum via the intraperitoneal (i.p.) route 24 hours prior to challenge infections with the test organism given i.p. (Beachey et al., "Human immune response to immunization with a structurally defined polypeptide fragment of streptococcal M protein," *J. Exp. Med.* 30 150:862-877, 1979). For each test organism, groups of 25 mice receive either preimmune or immune serum. The animals are then divided into 5 groups of 5 mice

each and 10-fold increasing challenge doses of virulent streptococci are given to each subgroup. After 7 days of observation, the LD₅₀ is calculated for each serotype tested.

Direct mouse protection tests are similarly performed except that mice
5 are actively immunized with M protein vaccine prior to the challenge infections. Each mouse receives 25-50ug vaccine in alum given intramuscularly (i.m.) at time 0, 4 weeks, and 8 weeks. Challenge infections are performed ten weeks after the first injection. Control animals are sham immunized with alum alone. The LD₅₀ is calculated and significance is determined using Fisher's exact test.

10 B. Protection

In order to show directly the protective efficacy of opsonic antibodies evoked by the hexavalent vaccine, mice were immunized with the vaccine adsorbed to ALUM and then challenged with two of the serotypes represented in the vaccine. Female outbred white Swiss mice were immunized via the I.M. route in the hind leg
15 according to the following schedule: time 0, 25ug; 3 weeks, 25 ug; 6 weeks, 50ug; and 13 weeks, 50ug. Challenge experiments were performed on the 20 immunized mice and 20 control, unimmunized mice (Table 1.) The challenge strains were types 24 and 19, with the reasoning that the M24 peptide is the largest fragment in the hexavalent protein and is reiterated and the M19 fragment is one of two that are only
20 35 amino acids long. These two fragments should reflect the range of protective immunogenicity of the hexavalent protein. Interperitoneal challenge of mice with virulent streptococci is the most stringent laboratory assay for opsonic antibodies.

In this experiment, two groups of ten mice each were challenged with an inoculum that approximated the LD₇₀-LD₁₀₀ for each serotype, which was 2x10⁴
25 CFU. The challenge experiments were begun 15 weeks after the first dose of vaccine was administered and deaths were recorded for 10 days. The mice that were immunized with the hexavalent vaccine and challenged with type 24 streptococci were significantly protected from death compared to the control group (p=.0001). The mice challenged with type 19 streptococci were protected by vaccination, but the
30 level was not statistically significant (p=.15). Had the challenged group been twice the size, the same level of protection would have resulted in a statistically significant

survival rate. When the survival of the entire immunized group of mice is analyzed, the level of protection was highly significant ($p=.0002$).

5 **Table 1. Protective immunogenicity of the hexavalent vaccine in mice that were challenged i.p. with virulent type 24 and type 19 streptococci**

Group	#Dead/#Survived of Mice Challenged (% survival)		
	Type 24	Type 19	Total
Immunized mice	0/10 (100)	4/6 (60)	4/16 (80) p=.0002*
Control mice	9/1 (10)	7/3 (30)	16/4 (20)

*p value was calculated using the Fisher exact test.

EXAMPLE 5

ASSAYS FOR TISSUE-CROSSREACTIVE ANTIBODIES

10 To assure that none of the M protein vaccines evokes tissue-crossreactive antibodies, indirect immunofluorescence assays are performed using frozen sections of human heart, kidney, and brain (Dale, J.B. and Beachey E.H., "Protective antigenic determinant of streptococcal M protein shared with sarcolemmal membrane protein of human heart," *J. Exp. Med.* 156:1165-1176, 1982).

15 Thin sections of tissue obtained at autopsy (4um) are prepared on microscope slides and stored in a sealed box at -70°C until use. Test serum is diluted 1:5 in PBS and dropped onto the tissue section. Control slides are made with preimmune serum and PBS. The slides are incubated at ambient temperature for 30 minutes and then washed three times in PBS in a slide holder. Fluorescein-labeled goat anti-

20 IgG/IgM/IgA is diluted 1:40 in PBS and dropped onto the slides which are again washed, dried, and mounted with 1% Gelvetol and a coverslip. Fluorescence is detected using a Zeiss Axiophot microscope equipped with a xenon light source. Immunofluorescence is recorded using a scale of 0-4+, with 0 being no fluorescence and 4+ being that obtained with a standard, positive antiserum raised in rabbits

25 against whole type 5 M protein (Dale, J.B. and Beachey, E.H., "Multiple heart-cross-reactive epitopes of streptococcal M proteins," *J. Exp. Med.* 161:113-122, 1985).

EXAMPLE 6**COMPARISON OF THE IMMUNOGENICITY OF A HEXAVALENT VACCINE**

5

DELIVERED IN ALUM VERSUS FREUND'S ADJUVANT

Three rabbits each were immunized with 100 ug doses of the hexavalent vaccine in either alum or CFA. Booster injections of the same dose were given at 4 and 8 weeks in either alum or saline, respectively. ELISA titers were 10 determined using the purified hexavalent protein as the solid phase antigen (Figure 3). Sera from the animals that received the hexavalent vaccine in alum had antibody titers that were equal to or greater than the sera from rabbits that received the same dose in CFA. In a subsequent experiment, three rabbits were immunized I.M. with 15 100 ug of the hexavalent vaccine in saline alone according the same schedule. None of these rabbits developed significant antibody titers against either the immunogen or the respective pep M proteins (data not shown). These data indicate that alum is a suitable and necessary adjuvant for the multivalent vaccine and is equal to the adjuvant activity of CFA in combination with the hexavalent protein.

20

EXAMPLE 7**PROTECTIVE IMMUNOGENICITY OF THE COMPONENT SUBUNITS OF A HEXAVALENT
VACCINE**

One of the major goals of this study was to design a multivalent, 25 hybrid protein that retained the immunogenic properties of each M protein subunit. ELISAs were performed on sera obtained from the three rabbits immunized with the hexavalent vaccine in alum (Figure 4). In each case the ELISA antigen was the purified pepsin-extracted M protein. Thus, the assay measures only the antibodies evoked by the hexavalent protein that react with the native M protein and not the 30 antibodies that may be specific for the joining segments or conformations that are not present in the native M proteins. The hexavalent protein evoked significant levels of antibodies against each M protein represented in the vaccine construct (Figure 4).

Importantly, none of the antisera contained antibodies that crossreacted with human heart tissue or kidney tissue, as determined by indirect immunofluorescence assays (data not shown).

Sera from all three rabbits contained significant levels of opsonic antibodies against each serotype of group A streptococci represented in the vaccine (Figure 5). These results were confirmed by indirect bactericidal assays using one of the immune sera (Table 2). Taken together, the results indicate that the individual components of the hexavalent vaccine retain the conformation and immunogenicity necessary to elicit antibodies that react with the native M proteins on the surface of each respective serotype of group A streptococci.

Table 2. Indirect bactericidal assay of rabbit antiserum against the hexavalent M protein vaccine.

<u>Serotype</u>	<u>Inoculum(CFU)</u>	<u>CFU Surviving 3 hr rotation: Percent Reduction</u>		
		<u>Preimmune</u>	<u>Immune</u>	
24	12	2890	0	100
5	11	3260	0	100
6	6	2640	0	100
19	6	1580	0	100
1	8	2670	490	82
3	11	1720	10	99

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

CLAIMS

I claim:

1. An immunogenic fusion polypeptide which stimulates an immune response against group A streptococci, comprising:

(a) at least two immunogenic polypeptides from a group A streptococci of at least 10 amino acids in length which are capable of stimulating an immune response against group A streptococci; and

(b) a peptide C terminal to the immunogenic polypeptide which protects the immunogenicity of the immunogenic portion, wherein the C-terminal peptide is not required to stimulate an immune response against group A streptococci.

2. The polypeptide according to claim 1 wherein one of said immunogenic polypeptides is obtained from a serotype 5 group A streptococci.

3. The polypeptide according to claim 1 wherein one of said immunogenic polypeptides is obtained from a serotype 6 group A streptococci.

4. The polypeptide according to claim 1 wherein one of said immunogenic polypeptides is obtained from a group A streptococci serotype selected from the group consisting of 1, 1.1, 2, 3, 4, 11, 12, 13, 14, 18, 19, 22, 24, 28, 30, 48, 49, 52 and 56.

5. A vaccinating agent for promoting an immune response against group A streptococci, comprising:

(a) at least two immunogenic polypeptides from a group A streptococci of at least 10 amino acids in length which are capable of stimulating a protective immune response against group A streptococci; and

(b) a peptide C terminal to the immunogenic polypeptide which protects the immunogenicity of the immunogenic portion, wherein the C-terminal peptide is not required to stimulate an immune response against group A streptococci.

6. The vaccinating agent according to claim 5 wherein one of said immunogenic polypeptides is obtained from a serotype 5 group A streptococci.

7. The vaccinating agent according to claim 5 wherein one of said immunogenic polypeptides is obtained from a serotype 6 group A streptococci.

8. The vaccinating agent according to claim 5 wherein one of said immunogenic polypeptides is obtained from a group A streptococci serotype selected from the group consisting of 1, 1.1, 2, 3, 4, 11, 12, 13, 14, 18, 19, 22, 24, 28, 30, 48, 49, 52 and 56.

9. The vaccinating agent according to claim 5, further comprising an adjuvant.

10. The vaccinating agent according to claim 9 wherein the adjuvant is alum or Freund's adjuvant.

11. A method for vaccinating a host against group A streptococci infections, comprising administering a vaccinating agent according to any one of claims 5 to 7.

FIGURE 1

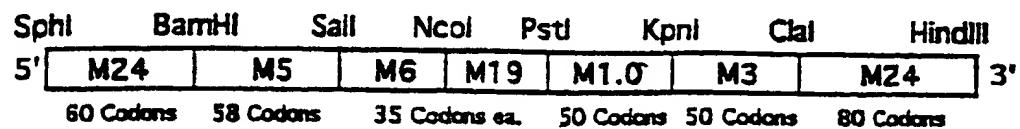


FIGURE 2

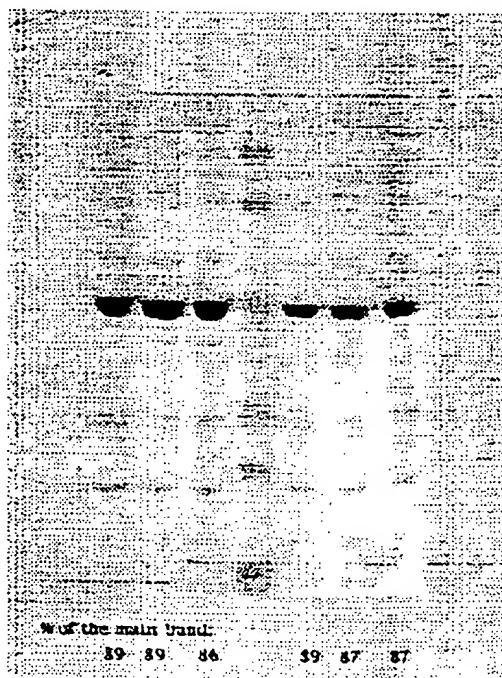


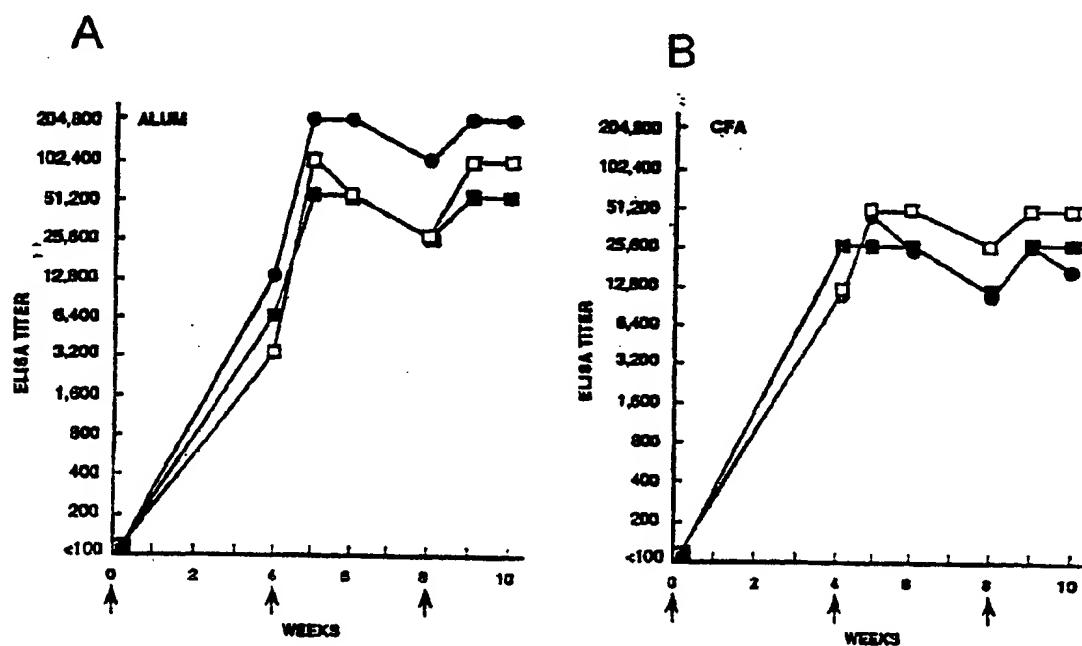
FIGURE 3

FIGURE 4

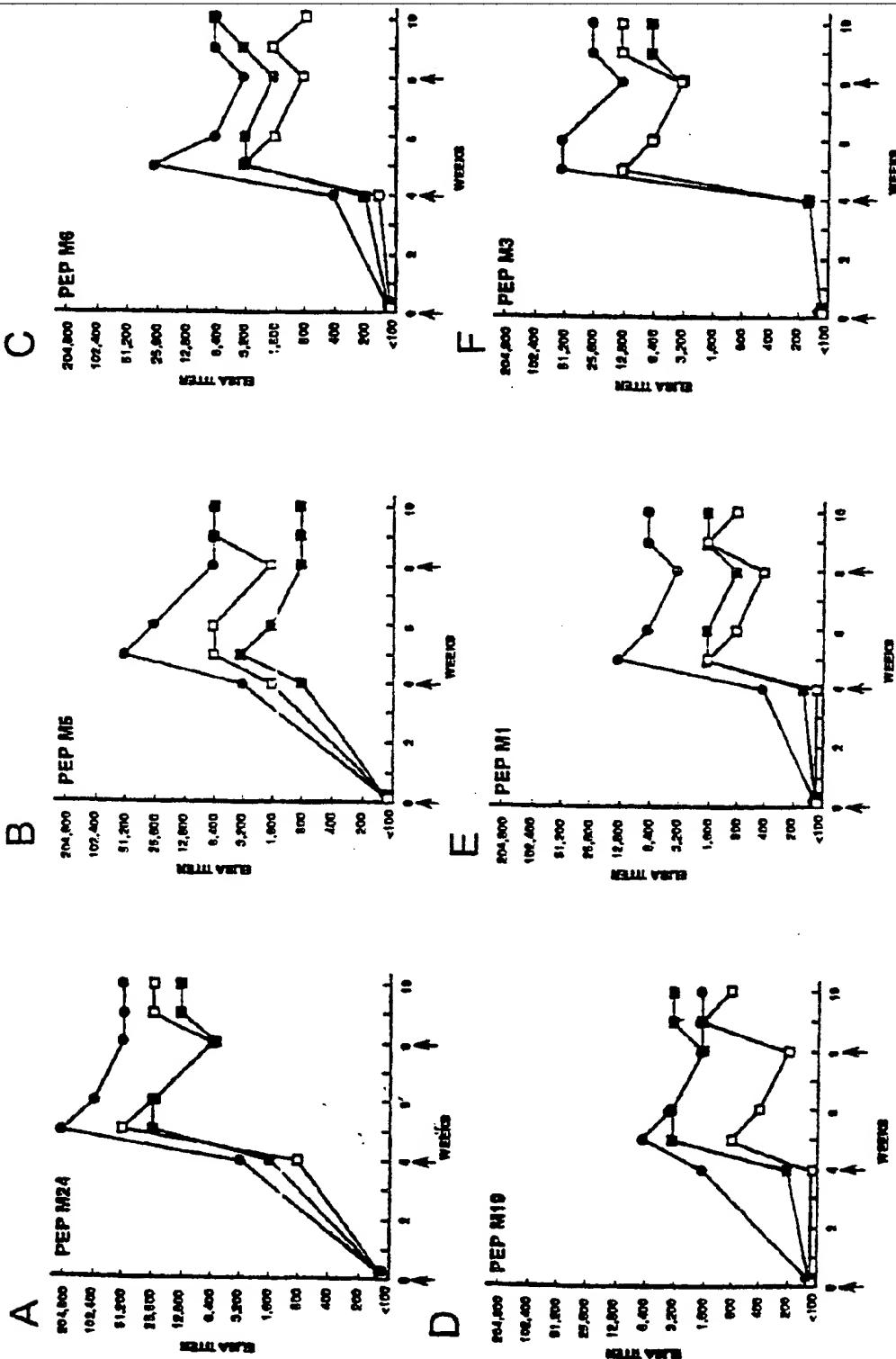


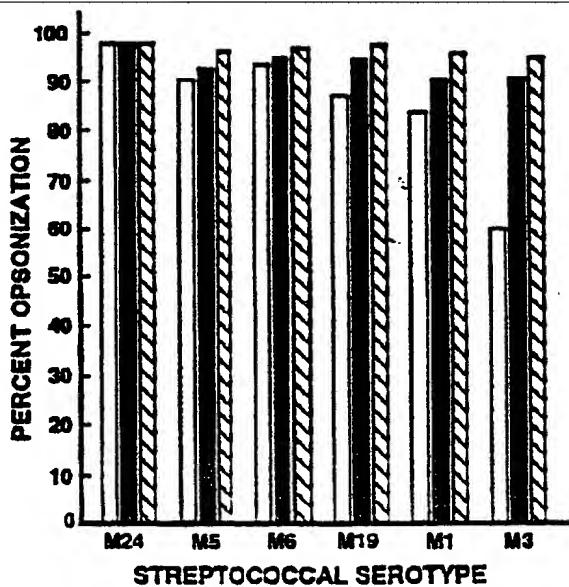
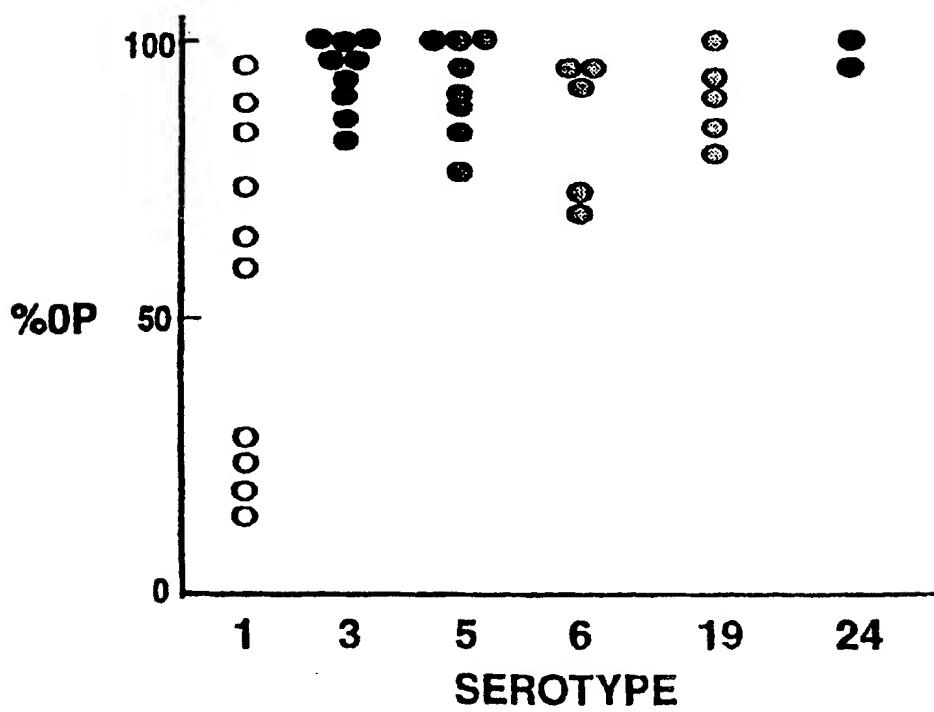
FIGURE 5**FIGURE 6**

FIGURE 7A**HEXAVALENT M. PROTEIN VACCINE SEQUENCE**

10 20 30 40 50 60 70

GCA TGC ATG GTC GCG ACT AGG TCT CGG ACA GAT ACT CTC GAA AAA GTC CAA CAA CGT CCT GAC AAG TTT CGC ATA
Ala Cys Met Val Ala Thr Arg Ser Gln Thr Asp Thr Leu Glu Lys Val Gln Glu Arg Ala Asp Lys Phe Glu Ile
-SphI- M24----->

80 90 100 110 120 130 140 150

CRA AAC AAT ACC TTA AAA CCT AAC ACT AGT GAC TTA ACT TTT AAT ATA AAA GCG TTA AAA GAT CAT AAT GAT GAC
Glu Asn Asn Thr Leu Lys Leu Lys Asn Ser Asp Leu Ser Phe Asn Asn Lys Ala Leu Lys Asp His Asn Asp Glu

160 170 180 190 200 210 220

TTA ACT GAA GAG TTG AGT AAT CCT AAA GAG AAA CTA CGT GGA TCC CCC GTC GIG ACT AGG CGT ACA ATA AAT GAC CGG
Leu Thr Glu Glu Leu Ser Asn Ala Lys Glu Lys Leu Arg Gly Ser Ala Val Thr Arg Gly Thr Ile Asn Asp Pro
-BamHI- MS----->

230 240 250 260 270 280 290 300

CAA AGA GCA AAA GAA CCT CTT GAC AAG TAT GAG CTA GAA AAC CAT GAC TTA AAA ACT AAG AAT GAA CGG TTA AAA
Gln Arg Ala Lys Glu Ala Leu Asp Lys Tyr Glu Leu Glu Asn His Asp Leu Lys Thr Lys Asn Glu Gly Leu Lys

310 320 330 340 350 360 370

ACT GAG AAT GAA CGG TTA AAA ACT GAG AAT GAA CGG TTA AAA ACT GAG AAT GAA CGG TTA AAA ACT GAG GTC GAC
Thr Glu Asn Glu Gly Leu Lys Thr Glu Asn Glu Gly Leu Lys Thr Glu Asn Glu Gly Leu Lys Thr Glu Val Asp
-SalI-

380 390 400 410 420 430 440 450

AGA GTC TTT CCT ACC CGG ACC GTC GAA AAC CGG GAC AAA GCA CCA GAA CCT CCT AAC AAG TAT GAC GTC GAG AAC
Arg Val Phe Pro Arg Gly Thr Val Glu Asn Pro Asp Lys Ala Arg Glu Leu Leu Asn Lys Tyr Asp Val Glu Asn
M6----->

460 470 480 490 500 510 520

TCT ATG TTA CAA GCT AAT AAT GAC AAG TTA CCA TGG AGA GIG CGT TAT ACT AGG CAT ACC CCA GAA GAT AAG CTA
Ser Met Leu Gln Ala Asn Asp Lys Leu Pro Trp Arg Val Arg Tyr Thr Arg His Thr Pro Glu Asp Lys Leu
-NcoI- M19----->

530 540 550 560 570 580 590 600

AAA AAA ATT ATT GAC GAT CCT GTC GCA AAA GAA CAT GAA TTA CAA CAA CAG AAT GAG AAC TTA TCT CTG CGG AAC
Lys Lys Ile Ile Asp Asp Leu Asp Ala-Lys Glu His Glu Leu Gln Gln Asn Glu Lys Leu Ser Leu Gln Asn
-PstI- M10----->

610 620 630 640 650 660 670

GCT GAT CGT AAT CCT ACC GAA GTT ATA GAA GAT CCT CCA CGA AAC AAT CCC GCA ATA CAA AAT ATA CST TTA CGT
Gly Asp Gly Asn Pro Arg Glu Val Ile Glu Asp Leu Ala Ala Asn Pro Ala Ile Gln Asn Ile Arg Leu Arg
----->

FIGURE 7B

680 690 700 710 720 730 740 750
 CGC GAA AAC AAG GAC TTA AAA GCG AGA TTA GCG AAT GCA ATG GAA GCA GCA AGA CAT TTT AAG AGA GCT GGT
 His Glu Asn Lys Asp Leu Lys Ala Arg Leu Glu Asn Ala Met Glu Val Ala Gly Arg Asp Phe Lys Arg Ala Gly
 RpnL
 760 770 780 790 800 810 820
 ACC TTG TTA GAT CAG GTT ACA CAA TTA TAT ACT AAA CAT AAT AGT AAT TAC CAA CAA TAT AAT GCA CAA GCT GGC
 Thr Leu Leu Asp Cln Val Thr Gln Leu Tyr Thr Lys His Asn Ser Asn Tyr Gln Gln Tyr Asn Ala Gln Ala Gly
 --- M3 ----->
 830 840 850 860 870 880 890 900
 AGA CTT CGC CTG AGA CAA AAG GCT GAA TAT CTA AAA GGC CTT AAT GAT TGG CCT GAG AGG CTG TTA CAA GAG TTA
 Arg Leu Asp Leu Arg Gln Lys Ala Glu Tyr Leu Lys Gly Leu Asn Asp Trp Ala Glu Arg Leu Gln Glu Leu
 910 920 930 940 950 960 970
 AAT ATC GAT GTC CGG ACT ACG TCT CGG ACA GAT ACT CTG GAA AAA GTC CAA GAA GCG CCT GCT CGC AAC TTT GAG ATA
 Asn Ile Asp Val Ala Thr Arg Ser Gln Thr Asp Thr Leu Glu Lys Val Gln Glu Arg Ala Asp Lys Phe Glu Ile
 -Clai- M24 ----->
 980 990 1000 1010 1020 1030 1040 1050
 GAA AAC AAT ACG TTA AAA CTT AAG AAT AGT GRC TTA AGT TTT AAT AAT AAA GCG TTA AAA GAT GAT AAT GAT GAG
 Glu Asn Asn Thr Leu Lys Leu Lys Asn Ser Asp Leu Ser Phe Asn Asn Lys Ala Leu Lys Asp His Asn Asp Glu
 1060 1070 1080 1090 1100 1110 1120
 TTA ACT GAA GAG TTG AGT AAT CCT AAA GAG AAA CTA CCT AAA AAT GAT AAA TCA CTA TCT GAA AAA GCT AGT AAA
 Leu Thr Glu Leu Ser Asn Ala Lys Glu Lys Leu Arg Lys Asn Asp Lys Ser Leu Ser Glu Lys Ala Ser Lys
 1130 1140 1150
 ATT CAA GAA TTA GAG GCA CGT AAG TAA AAG CTT
 Ile Gln Glu Leu Glu Ala Arg Lys *** Lys Leu
 Stoc HindIII

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Dale, James B.
- (ii) TITLE OF INVENTION: GROUP A STREPTOCOCAL VACCINES
- (iii) NUMBER OF SEQUENCES: 16
- (iv) CORRESPONDENCE ADDRESS:
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 - (D) STATE: Washington
 - (E) COUNTRY: USA
 - (F) ZIP: 98104
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: US
 - (B) FILING DATE: 12-SEP-1997
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: McMasters, David D.
 - (B) REGISTRATION NUMBER: 33,963
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- (ix) TELECOMMUNICATION INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 36 base pairs
 - (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGGGGGGCAT CGGTCGCGAC TAGGTCTCAG ACAGAT

36

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGGGGGGGAT CCACGTAGTT TCTCTTTAGC

30

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGGGGGGAT CCGCCGTGAC TAGGGGTACA

30

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGGGGGGGTCG ACCTCAGTTT TTAACCCCTTC

30

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGGGGGGGTCG ACAGAGTGTT TCCTAGGGGG

30

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGGGGGGCCAT GGTAACTTGT CATTATTAGC

30

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGGGGGGCCAT GGAGAGTGCG TTATACTAGG

30

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGGGGGCTGC AGAGATAACT TCTCATTCTG

30

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGGGGGCTGC AGAACGGTGA TGGTAATCCT

30

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GGGGGGGGTA CCAGCTCTCT TAAAATCTCT

30

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGGGGGGGTA CCTTGTTAGA TCAGGTTACA

30

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGGGGGATCG ATATTTAACT CTTGTAACAG

30

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGGGGGATCG ATGTCGCGAC TAGGTCTCAG

30

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GGGGGGAAGC TTTTACTTAC GTGCCTCTAA TTC

33

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1158 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ix) FEATURE:

(A) NAME/KEY: CDS
 (B) LOCATION: 1..1149

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GCA TGC ATG GTC GCG ACT AGG TCT CAG ACA GAT ACT CTG GAA AAA GTA	48
Ala Cys Met Val Ala Thr Arg Ser Gln Thr Asp Thr Leu Glu Lys Val	
1 5 10 15	
CAA GAA CGT GCT GAC AAG TTT GAG ATA GAA AAC AAT ACG TTA AAA CTT	96
Gln Glu Arg Ala Asp Lys Phe Glu Ile Glu Asn Asn Thr Leu Lys Leu	
20 25 30	
AAG AAT AGT GAC TTA AGT TTT AAT AAT AAA GCG TTA AAA GAT CAT AAT	144
Lys Asn Ser Asp Leu Ser Phe Asn Asn Lys Ala Leu Lys Asp His Asn	
35 40 45	
GAT GAG TTA ACT GAA GAG TTG AGT AAT GCT AAA GAG AAA CTA CGT GGA	192
Asp Glu Leu Thr Glu Leu Ser Asn Ala Lys Glu Lys Leu Arg Gly	
50 55 60	
TCC GCC GTG ACT AGG GGT ACA ATA AAT GAC CCG CAA AGA GCA AAA GAA	240
Ser Ala Val Thr Arg Gly Thr Ile Asn Asp Pro Gln Arg Ala Lys Glu	
65 70 75 80	
GCT CTT GAC AAG TAT GAG CTA GAA AAC CAT GAC TTA AAA ACT AAG AAT	288
Ala Leu Asp Lys Tyr Glu Leu Glu Asn His Asp Leu Lys Thr Lys Asn	
85 90 95	
GAA GGG TTA AAA ACT GAG AAT GAA GGG TTA AAA ACT GAG AAT GAA GGG	336
Glu Gly Leu Lys Thr Glu Asn Glu Gly Leu Lys Thr Glu Asn Glu Gly	
100 105 110	
TTA AAA ACT GAG AAT GAA GGG TTA AAA ACT GAG GTC GAC AGA GTG TTT	384
Leu Lys Thr Glu Asn Glu Gly Leu Lys Thr Glu Val Asp Arg Val Phe	
115 120 125	
CCT AGG GGG ACG GTA GAA AAC CCG GAC AAA GCA CGA GAA CTT CTT AAC	432
Pro Arg Gly Thr Val Glu Asn Pro Asp Lys Ala Arg Glu Leu Leu Asn	

130	135	140	
AAG TAT GAC GTA GAG AAC TCT ATG TTA CAA GCT AAT AAT GAC AAG TTA Lys Tyr Asp Val Glu Asn Ser Met Leu Gln Ala Asn Asn Asp Lys Leu 145 150 155 160			480
CCA TGG AGA GTG CGT TAT ACT AGG CAT ACG CCA GAA GAT AAG CTA AAA Pro Trp Arg Val Arg Tyr Thr Arg His Thr Pro Glu Asp Lys Leu Lys 165 170 175			528
AAA ATT ATT GAC GAT CTT GAC GCA AAA GAA CAT GAA TTA CAA CAA CAG Lys Ile Ile Asp Asp Leu Asp Ala Lys Glu His Glu Leu Gln Gln Gln 180 185 190			576
AAT GAG AAG TTA TCT CTG CAG AAC GGT GAT GGT AAT CCT AGG GAA GTT Asn Glu Lys Leu Ser Leu Gln Asn Gly Asp Gly Asn Pro Arg Glu Val 195 200 205			624
ATA GAA GAT CTT GCA GCA AAC AAT CCC GCA ATA CAA AAT ATA CGT TTA Ile Glu Asp Leu Ala Ala Asn Asn Pro Ala Ile Gln Asn Ile Arg Leu 210 215 220			672
CGT CAC GAA AAC AAG GAC TTA AAA GCG AGA TTA GAG AAT GCA ATG GAA Arg His Glu Asn Lys Asp Leu Lys Ala Arg Leu Glu Asn Ala Met Glu 225 230 235 240			720
GTT GCA GGA AGA GAT TTT AAG AGA GCT GGT ACC TTG TTA GAT CAG GTT Val Ala Gly Arg Asp Phe Lys Arg Ala Gly Thr Leu Leu Asp Gln Val 245 250 255			768
ACA CAA TTA TAT ACT AAA CAT AAT AGT AAT TAC CAA CAA TAT AAT GCA Thr Gln Leu Tyr Thr Lys His Asn Ser Asn Tyr Gln Gln Tyr Asn Ala 260 265 270			816
CAA GCT GGC AGA CTT GAC CTG AGA CAA AAG GCT GAA TAT CTA AAA GGC Gln Ala Gly Arg Leu Asp Leu Arg Gln Lys Ala Glu Tyr Leu Lys Gly 275 280 285			864
CTT AAT GAT TGG GCT GAG AGG CTG TTA CAA GAG TTA AAT ATC GAT GTC Leu Asn Asp Trp Ala Glu Arg Leu Leu Gln Glu Leu Asn Ile Asp Val 290 295 300			912
GCG ACT AGG TCT CAG ACA GAT ACT CTG GAA AAA GTA CAA GAA CGT GCT Ala Thr Arg Ser Gln Thr Asp Thr Leu Glu Lys Val Gln Glu Arg Ala 305 310 315 320			960

GAC AAG TTT GAG ATA GAA AAC AAT ACG TTA AAA CTT AAG AAT AGT GAC	1008	
Asp Lys Phe Glu Ile Glu Asn Asn Thr Leu Lys Leu Lys Asn Ser Asp		
325	330	335
TTA AGT TTT AAT AAT AAA GCG TTA AAA GAT CAT AAT GAT GAG TTA ACT	1056	
Leu Ser Phe Asn Asn Lys Ala Leu Lys Asp His Asn Asp Glu Leu Thr.		
340	345	350
GAA GAG TTG AGT AAT GCT AAA GAG AAA CTA CGT AAA AAT GAT AAA TCA	1104	
Glu Glu Leu Ser Asn Ala Lys Glu Lys Leu Arg Lys Asn Asp Lys Ser		
355	360	365
CTA TCT GAA AAA GCT AGT AAA ATT CAA GAA TTA GAG GCA CGT AAG	1149	
Leu Ser Glu Lys Ala Ser Lys Ile Gln Glu Leu Glu Ala Arg Lys		
370	375	380
TAAAAAGCTT	1158	

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 383 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Ala Cys Met Val Ala Thr Arg Ser Gln Thr Asp Thr Leu Glu Lys Val
1 5 10 15

Gln Glu Arg Ala Asp Lys Phe Glu Ile Glu Asn Asn Thr Leu Lys Leu
20 25 30

Lys Asn Ser Asp Leu Ser Phe Asn Asn Lys Ala Leu Lys Asp His Asn
35 40 45

Asp Glu Leu Thr Glu Glu Leu Ser Asn Ala Lys Glu Lys Leu Arg Gly
50 55 60

Ser Ala Val Thr Arg Gly Thr Ile Asn Asp Pro Gln Arg Ala Lys Glu
65 70 75 80

Ala Leu Asp Lys Tyr Glu Leu Glu Asn His Asp Leu Lys Thr Lys Asn
85 90 95

Glu Gly Leu Lys Thr Glu Asn Glu Gly Leu Lys Thr Glu Asn Glu Gly
100 105 110

Leu Lys Thr Glu Asn Glu Gly Leu Lys Thr Glu Val Asp Arg Val Phe
115 120 125

Pro Arg Gly Thr Val Glu Asn Pro Asp Lys Ala Arg Glu Leu Leu Asn
130 135 140

Lys Tyr Asp Val Glu Asn Ser Met Leu Gln Ala Asn Asn Asp Lys Leu
145 150 155 160

Pro Trp Arg Val Arg Tyr Thr Arg His Thr Pro Glu Asp Lys Leu Lys
165 170 175

Lys Ile Ile Asp Asp Leu Asp Ala Lys Glu His Glu Leu Gln Gln Gln
180 185 190

Asn Glu Lys Leu Ser Leu Gln Asn Gly Asp Gly Asn Pro Arg Glu Val
195 200 205

Ile Glu Asp Leu Ala Ala Asn Asn Pro Ala Ile Gln Asn Ile Arg Leu
210 215 220

Arg His Glu Asn Lys Asp Leu Lys Ala Arg Leu Glu Asn Ala Met Glu
225 230 235 240

Val Ala Gly Arg Asp Phe Lys Arg Ala Gly Thr Leu Leu Asp Gln Val
245 250 255

Thr Gln Leu Tyr Thr Lys His Asn Ser Asn Tyr Gln Gln Tyr Asn Ala
260 265 270

Gln Ala Gly Arg Leu Asp Leu Arg Gln Lys Ala Glu Tyr Leu Lys Gly
275 280 285

Leu Asn Asp Trp Ala Glu Arg Leu Leu Gln Glu Leu Asn Ile Asp Val
290 295 300

Ala Thr Arg Ser Gln Thr Asp Thr Leu Glu Lys Val Gln Glu Arg Ala
305 310 315 320

Asp Lys Phe Glu Ile Glu Asn Asn Thr Leu Lys Leu Lys Asn Ser Asp
325 330 335

Leu Ser Phe Asn Asn Lys Ala Leu Lys Asp His Asn Asp Glu Leu Thr
340 345 350

Glu Glu Leu Ser Asn Ala Lys Glu Lys Leu Arg Lys Asn Asp Lys Ser
355 360 365

Leu Ser Glu Lys Ala Ser Lys Ile Gln Glu Leu Glu Ala Arg Lys
370 375 380

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/19100

A. CLASSIFICATION OF SUBJECT MATTER	IPC 6 C12N15/31 C12N15/62 C07K14/315 A61K39/09 A61K39/39
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>BEACHEY E H ET AL: "PROTECTIVE IMMUNOGENICITY AND T LYMPHOCYTE SPECIFICITY OF A TRIVALENT HYBRID PEPTIDE CONTAINING NH2-TERMINAL SEQUENCES OF TYPES 5, 6, AND 24 M PROTEINS SYNTHESIZED IN TANDEM" JOURNAL OF EXPERIMENTAL MEDICINE, vol. 166, no. 3, 1 September 1987, pages 647-656, XP000618953 see page 647, last paragraph; figure 1</p>	1-3,5-7, 11
Y	<p>DALE J B ET AL: "Recombinant, octavalent group A streptococcal M protein vaccine" VACCINE, vol. 14, no. 10, July 1996, page 944-948 XP004057623 cited in the application see figure 1</p>	4,8-10
		-/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"S" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

10 February 1999

24/02/1999

Name and mailing address of the ISA

Authorized officer

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Esben, J

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/19100

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DALE J B ET AL: "RECOMBINANT TETRAVALENT GROUP A STREPTOCOCCAL M PROTEIN VACCINE" JOURNAL OF IMMUNOLOGY, vol. 151, no. 4, 15 August 1993, pages 2188-2194, XP000645174 cited in the application see figure 1; tables III,V ---	4,8-10
Y	BEACHEY E H ET AL: "OPSONIC ANTIBODIES EVOKED BY HYBRID PEPTIDE COPIES OF TYPES 5 AND 24 STREPTOCOCCAL M PROTEINS SYNTHESIZED IN TANDEM" JOURNAL OF EXPERIMENTAL MEDICINE, vol. 163, no. 6, June 1986, pages 1451-1458, XP000645526 see page 1456, last paragraph; figure 1 ---	4,8-10
Y	WO 94 06421 A (UNIV TENNESSEE RES CORP) 31 March 1994 see page 12 - page 13; figures 1-13 ---	4,8-10
Y	WISTEDT A C ET AL: "Identification of a plasminogen-binding motif in PAM, a bacterial surface protein" MOLECULAR MICROBIOLOGY, vol. 18, 1995, pages 569-578, XP002092777 GB see page 572, right-hand column -----	4,8-10

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/19100

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim 11 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/19100

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9406421 A	31-03-1994	AU 5128493 A	CA 2123579 A	12-04-1994 31-03-1994